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(57) Abstract

A human BMP-10 polypeptide and DNA (RNA) encoding such polypeptide and a procedure for producing such polypeptide by recombinant techniques is disclosed. Also disclosed are methods for utilizing such polypeptide for stimulating de novo bone synthesis. Diagnostic assays for identifying mutations in nucleic acid sequence encoding a polypeptide of the present invention and for detecting altered levels of the polypeptide of the present invention for detecting diseases, for example cancer, are also disclosed.

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# BONE MORPHOGENIC PROTEIN-10

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention is Bone Morphogenic Protein-10 "BMP-10". The invention also relates to inhibiting the action of such polypeptides.

There are approximately 600,000 nonunion bone fractures a year in the United States alone. BMP may be used to induce bone and/or cartilage formation and is therefore useful in wound healing and tissue repair. BMP may be used for treating a number of bone defects and periodontal disease and various types of wounds.

A 32-36 kDa osteogenic protein purified from bovine bone matrix is composed of dimers of two members of the transforming growth factor-beta super family, the bovine equivalent of human osteogenic protein-1 and bone morphogenic protein-2a. It is reported that recombinant human osteogenic protein-1 (HOP-1) induces new bone formation *in vivo* with a specific activity compatible with natural bovine osteogenic protein and stimulates osteoblast proliferation and

differentiation *in vitro* (Sampath, T.K., et al., J. Biol. Chem., 267:20352-62 (1992)). The recombinant human osteogenic protein-1 (HOP-1) was produced in mammalian cells as a processed mature disulfide-linked homodimer with an apparent molecular weight of 36,000. The evaluation of HOP-1 effects on cell growths and collagen synthesis in rat osteoblast-enriched bone cell cultures showed that both cell proliferation and collagen synthesis were stimulated in a dose-dependent manner and increased three-fold in response to 40ng of HOP-1/ml.

It has also recently been shown that ectopic expression of DVR-4 (Bone Morphogenetic Protein-4) induces amphibian embryos to develop with an overall posterior and/or ventral character, and that DVR-4 induces ventral types of mesoderm in animal explants. DVR-4 is therefore the first molecule reported both to induce posteroventral mesoderm and to counteract dorsalizing signals such as activin, (Jones, C.M. et al, Development, 115:639-47 (1992)).

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is BMP-10, as well as fragments, analogs and derivatives thereof. The polypeptide of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided polynucleotides (DNA or RNA) which encode such polypeptides.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides for therapeutic purposes, for example, for the promotion of *de novo* bone formation during surgical insertion of protheses, for the treatment of non-union bone fractures, and for treatment of osteoporosis and periodontal disease.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with another aspect of the present invention, there are provided agonists which mimic the polypeptide of the present invention and bind to the receptors.

In accordance with yet a further aspect of the present invention, there is also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for detecting diseases or susceptibility to diseases related to mutations in the nucleic acid sequences encoding a polypeptide of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, for example, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

FIG. 1 depicts the cDNA and corresponding deduced amino acid sequence of the mature BMP-10 polypeptide. The amino acid sequence is represented by the standard three letter code for amino acids.

The polypeptide of the present invention has a putative leader sequence comprising the initial 33 amino acids. The

active portion of the protein comprises from amino acid 344 to amino acid 478 of Figure 1 (SEQ ID NO:2).

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. _____ on June 2, 1995.

A polynucleotide encoding a polypeptide of the present invention was discovered in a fetal lung cDNA library. It contains an open reading frame encoding a mature polypeptide of 378 amino acids and shows 80 % sequence identity to the BMP-3a gene product. The polypeptide is a member of the bone morphogenic protein family which is a subfamily of the transforming growth factor Beta (TGF-$\beta$) superfamily.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of Figure 1 (SEQ ID NO:1) or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and

non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID NO:2) or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1) or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be

fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other

cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 80%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for

the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a BMP-10 polypeptide which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1a or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 80% similarity (preferably at least 80% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional

nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the BMP-10 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda $P_L$ promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or mor selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may

be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda $P_R$, $P_L$ and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second

Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of

replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a

compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and lectin chromatography. It is preferred to have low concentrations (approximately 0.15-5 mM) of calcium ion present during purification. (Price et al., J. Biol. Chem., 244:917 (1969)). Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The BMP-10 may be employed to promote de novo bone formation which may be used in the treatment of periodontal disease and other bone defects of the oral cavity.

BMP-10 may also be used during surgical insertion of protheses. In hip replacements, knee replacements and other surgical insertion of protheses, the prothesis is held in place by surgical cement. The cement eventually loosens, however, making it necessary to perform another surgery. This second surgery is a much more difficult procedure and is responsible for surgeons reluctantance to insert protheses in young people. BMP-10 may, therefore, be used to coat the prothesis before insertion which results in bone formation around the prothesis, making a stronger union and allowing for the use of less cement.

BMP-10 may also be employed in the treatment of osteoporosis, which is characterized by excessive bone resorption resulting in thin and brittle bones. BMP-10 would stimulate bone formation to help alleviate this condition.

This invention provides a method for identification of the receptor for the polypeptide of the present invention. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptide of the present invention, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptide of the present invention. Transfected cells which are grown on glass slides are exposed to labeled BMP-10. BMP-10 can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to auto-radiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process,

eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled ligand can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing the ligand-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention also provides a method for detecting compounds which mimic the activity of the polypeptide of the present invention. The method for determining whether a ligand can bind to the BMP-10 receptor comprises transfecting a cell population (one presumed not to contain the receptor) with the appropriate vector expressing the receptor, such that the cell will now express the receptor. A suitable response system is obtained by transfection of the DNA into a suitable host containing the desired second messenger pathways including cAMP, ion channels, phosphoinositide kinase, or calcium response. Such a transfection system provides a response system to analyze the activity of various ligands exposed to the cell. Ligands chosen could be identified through a rational approach by taking known ligands that interact with similar types of receptors or using small molecules, cell supernatants or extracts or natural products.

In another example, a mammalian cell or membrane preparation expressing the receptor is incubated with the compound. The ability of the compound to stimulate the response of a known second messenger system following interaction of compound and receptor would be measured. Such

second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

The polypeptides and agonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus,

Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or hetorologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X,

VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The polypeptides and agonists of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological

products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides and agonists of the present invention may be employed in conjunction with other therapeutic compounds.

BMP-10 is preferably used topically, however, when it is used systemically, the pharmaceutical compositions may be administered in a convenient manner such as by the oral, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, or intradermal routes. The amounts and dosage regimens of pharmaceutical compositions of BMP-10 administered to a subject will depend on a number of factors such as the mode of administration, the nature of the condition being treated, the body weight of the subject being treated and the judgment of the prescribing physician. Generally speaking, pharmaceutical compositions of BMP-10 are given, for example, in appropriate doses of at least about 10 μg/kg body weight and in most cases will not be administered in an amount in excess of about 8 mg/kg body weight, and preferably is given in doses of about 10 μg/kg body weight to about 1 mg/kg daily, taking into account the routes of administration, symptoms, etc.

Specifically, BMP-10 may be prepared as a gel matrix formulation and administered, for example, ectopically, which is preferably administered at the site of bone fracture at a dosage of 50 mg, by applying the matrix directly to the site of the fracture. This matrix may also be applied to protheses before insertion.

This invention is also related to the use of the gene of the present invention as a diagnostic. Detection of a mutated form of the gene will allow a diagnosis of a disease or a susceptibility to a disease which results from underexpression of BMP-10.

Individuals carrying mutations in the gene of the present invention may be detected at the DNA level by a

variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, including but not limited to blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding BMP-10 can be used to identify and analyze mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between the reference gene and genes having mutations may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different

positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of the polypeptide of the present invention in various tissues since an over-expression of the proteins compared to normal control tissue samples can detect the presence of lung cancer. Assays used to detect levels of the polypeptide of the present invention in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis and preferably an ELISA assay. An ELISA assay initially comprises preparing an antibody specific to the BMP-10 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is now removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumin. Next, the monoclonal

antibody is incubated in the dish during which time the monoclonal antibodies attached to any of the polypeptide of the present invention attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to the polypeptide of the present invention. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of the polypeptide of the present invention present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to the polypeptide of the present invention are attached to a solid support and labeled and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity of the polypeptide of the present invention in the sample.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than

one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA having at least 50 or 60 bases. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then

the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies

(Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger

volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

## Example 1

### Bacterial Expression and Purification of Human BMP-10

The DNA sequence encoding for human BMP-10 is initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end of the DNA sequence to synthesize insertion fragments. The 5' oligonucleotide primer 5'-GATCGGATCCAAAGCCCGGAGGAAGCAG-3' contains a Bam HI restriction

enzyme site followed by 18 nucleotides of BMP-10 coding sequence starting from amino acid 4 (Lys); the 3' sequence 5'-GTACTCTAGATCACCGGCAGGCACAGGTG-3' contains complementary sequences to an Xba I site, a translation stop codon and the last 16 nucleotides of BMP-10 coding sequence. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc., 9259 Eton Ave., Chatsworth, CA 91311, Catalog No. 33093). The plasmid vector encodes antibiotic resistance (Amp'), a bacterial origin of replication (ori), an IPTG-regulatable promoter/operator (P/O), a ribosome binding site (RBS), a 6-histidine tag (6-His) and restriction enzyme cloning sites. The pQE-9 vector was digested with Bam HI and Xba I and the insertion fragments were then ligated into the pQE-9 vector maintaining the reading frame initiated at the bacterial RBS. The ligation mixture was then used to transform the *E. coli* strain M15/rep4 available from Qiagen under the trademark m15/rep4. M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan'). Transformants are identified by their ability to grow on LB plates containing both Amp and Kan. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 μg/ml) and Kan (25 μg/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density of 600 ($O.D._{600}$) between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalacto pyranoside) was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 2-4 hours. Cells were then harvested by centrifugation (20 mins. at 6000Xg). The cell pellet was solubilized in the chaotropic agent 6 molar Guanidine HCl. After clarification, solubilized BMP-10 was purified from this solution by chromatography on a Nickel-

Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag. (Hochuli, E. et al., Genetic Engineering, Principles & Methods, 12:87-98 (1990). BMP-10 (95% pure) was eluted from the column in 6 molar GHCl pH 5.0. Protein renaturation out of GHCl can be accomplished by several protocols. (Jaenicke, R. and Rudolph R., Protein Structure - A Practical Approach, IRL Press, New York (1990). The pH 5.0 eluate was diluted and reapplied to a second nickel-chelate column. Bound protein was renatured by running a linear descending gradient of GnHCl. This allows for folding to occur on the column. The protein was then eluted with 250 μm imidazole pH 5.0.

Example 2

Tissue Distribution of BMP-10

Northern blot analysis was carried out to examine the levels of expression of BMP-10 in human tissues. Total cellular RNA samples were isolated with RNAzol™ B system (Biotecx Laboratories, Inc., 6023 South Loop East, Houston, TX 77033). About 10 ug of total RNA isolated from each human tissue specified was separated on 1% agarose gel and blotted onto a nylon filter (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50 ng DNA fragment. The labeled DNA was purified with a Select-G-50 column from 5' Prime -- 3 Prime, Inc., 5603 Arapahoe Road, Boulder, CO 80303. The filter was then hybridized with radioactive labeled full length BMP-10 gene at 1,000,000 cpm/ml in 0.5 M $NaPo_4$ and 7% SDS overnight at 65°C. The filters were washed twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS, the filters were then exposed at -70°C overnight with intensifying screen.

Example 3

Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and

separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the

gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

## Example 4

### Expression of recombinant BMP-10 in CHO cells

The vector pN346 is used for the expression of the BMP-10 protein. Plasmid pN346 is a derivative of the plasmid pSV2-dhfr [ATCC Accession No. 37146]. Both plasmids contain the mouse dhfr gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Lift Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F.W., Kellems, R.M.,

Bertino, J.R., and Schimke, R.T., 1978, J. Biol. Chem. 253:1357-1370, Hamlin, J.L. and Ma, C. 1990, Biochem. et Biophys. Acta, 1097:107-143, Page, M.J. and Sydenham, M.A. 1991, Biotechnology Vol. 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the dhfr gene it is usually co-amplified and over-expressed. It is state of the art to develop cell lines carrying more than 1,000 copies of the genes. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

Plasmid pN346 contains for the expression of the gene of interest a strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, et al., Molecular and Cellular Biology, March 1985, 438-447) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart et al., Cell 41:521-530, 1985). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Pvull, and Nrul. Behind these cloning sites the plasmid contains translational stop codons in all three reading frames followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for the expression, e.g., the human ß-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosome can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g. G418 plus methotrexate.

The plasmid pN346 was digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector was then isolated from a 1% agarose gel.

The DNA sequence encoding BMP-10, ATCC # _____ was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' GCGGATCCGCCATGGCTCATGT CCCC 3' and contains a BamHI restriction enzyme site (in bold) followed by 18 nucleotides resembling an efficient signal for translation (Kozak, M., supra) plus the first 12 nucleotides of the gene (the initiation codon for translation "ATG" is underlined.).

The 3' primer has the sequence 5' GCGGATCCTCAGGCGCA GGCTGTCCA 3' and contains the cleavage site for the restriction endonuclease BamHI and 18 nucleotides complementary to the 3' non-translated sequence of the gene.

The amplified fragments were isolated from a 1% agarose gel as described above and then digested with the endonuclease BglII and then purified again on a 1% agarose gel.

The isolated fragment and the dephosphorylated vector were then ligated with T4 DNA ligase. E.coli HB101 cells were then transformed and bacteria identified that contained the plasmid pN346 inserted in the correct orientation using the restriction enzymes BamHI. The sequence of the inserted gene was confirmed by DNA sequencing.

**Transfection of CHO-dhfr-cells**

Chinese hamster ovary cells lacking an active DHFR enzyme were used for transfection. 5 μg of the expression plasmid N346 were cotransfected with 0.5 μg of the plasmid pSVneo using the lipofectin method (Felgner et al., supra). The plasmid pSV2-neo contains a dominant selectable marker, the gene neo from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The

cells were seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells were trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) and cultivated from 10-14 days. After this period, single clones were trypsinized and then seeded in 6-well petri dishes using different concentrations of methotrexate (25, 50 nm, 100 nm, 200 nm, 400 nm). Clones growing at the highest concentrations of methotrexate were then transferred to new 6-well plates containing even higher concentrations of methotrexate (500 nM, 1 μM, 2 μM, 5 μM). The same procedure was repeated until clones grew at a concentration of 100 μM.

The expression of the desired gene product was analyzed by Western blot analysis and SDS-PAGE.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide encoding the polypeptide as set forth in Figure 1;

(b) a polynucleotide encoding the polypeptide comprising amino acid 344 to amino acid 478 as set forth in Figure 1;

(c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and

(d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 2 which encodes the polypeptide comprising amino acid 34 to 478 as set forth in Figure 1.

4. The polynucleotide of Claim 2 which encodes the polypeptide comprising amino acid 344 to 478 as set forth in Figure 1.

5. The polynucleotide of Claim 2 which encodes the polypeptide as set forth in Figure 1.

6. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide which encodes a mature polypeptide encoded by the DNA contained in ATCC Deposit No. _____;

(b) a polynucleotide which encodes a polypeptide expressed by the DNA contained in ATCC Deposit No. _____;

(c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and

(c) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).

7. A vector containing the DNA of Claim 2.

8. A host cell genetically engineered with the vector of Claim 7.

9. A process for producing a polypeptide comprising: expressing from the host cell of Claim 8 the polypeptide encoded by said DNA.

10. A process for producing cells capable of expressing a polypeptide comprising transforming or transfecting the cells with the vector of Claim 7.

11. A polypeptide comprising a member selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of Figure 1 and fragments, analogs and derivatives thereof; (ii) a polypeptide comprising amino acid 344 to amino acid 478 of Figure 1; and (iii) a polypeptide encoded by the cDNA of ATCC Deposit No. _____ and fragments, analogs and derivatives of said polypeptide.

12. A compound effective as an agonist for the polypeptide of claim 11.

13. A compound effective as an antagonist against the polypeptide of claim 11.

14. A method for the treatment of a patient having need of BMP-10 comprising: administering to the patient a

therapeutically effective amount of the polypeptide of claim 11.

15. The method of Claim 14 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

16. A method for the treatment of a patient having need of BMP-10 comprising: administering to the patient a therapeutically effective amount of the compound of claim 12.

17. A method for the treatment of a patient having need to inhibit BMP-10 comprising: administering to the patient a therapeutically effective amount of the antagonist of Claim 13.

18. A process for diagnosing a disease or a susceptibility to a disease related to expression of the polypeptide of claim 11 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

19. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 11 in a sample derived from a host.

20. A method for identifying compounds which bind to and activate or inhibit a receptor for the polypeptide of claim 11 comprising:

contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to

said receptor, with a compound to be screened under conditions to permit binding to the receptor; and

determining whether the compound binds to and activates or inhibits the receptor by detecting the presence or absence of a signal generated from the interaction of the compound with the receptor.

# FIG. 1A

```
1      CCTCACCACGCCATGGCTCATGTCCCCGCTCGGACCAGCCCGGGACCCGGGCCCCAGCTG
1                  M  A  H  V  P  A  R  T  S  P  G  P  G  P  Q  L
61     CTGCTGCTGCTGCTGCCGTTGTTTCTGCTGTTGCTCCGGGATGTGGCCGGCAGCCACAGG
17     L  L  L  L  L  P  L  F  L  L  L  L  R  D  V  A  G  S  H  R
121    GCCCCCGCCTGGTCCGCACTGCCCGCGGCCGCCGACGGCCTGCAGGGGGACAGGGATCTC
37     A  P  A  W  S  A  L  P  A  A  A  D  G  L  Q  G  D  R  D  L
181    CAGCGGCACCCTGGGGACGCGGCCGCCACGTTGGGCCCCAGCGCCCAGGACATGGTCGCT
57     Q  R  H  P  G  D  A  A  A  T  L  G  P  S  A  Q  D  M  V  A
241    GTCCACATGCACAGGCTCTATGAGAAGTACAGCCGGCAGGGCGCGCGGCCGGGAGGGGGC
77     V  H  M  H  R  L  Y  E  K  Y  S  R  Q  G  A  R  P  G  G  G
301    AACACGGTCCGCAGCTTCAGGGCCAGGCTGGAAGTGGTCGACCAGAAGGCCGTGTATTTC
97     N  T  V  R  S  F  R  A  R  L  E  V  V  D  Q  K  A  V  Y  F
361    TTCAACCTGACTTCCATGCAAGACTCGGAAATGATCCTTACGGCCACTTTCCACTTCTAC
117    F  N  L  T  S  M  Q  D  S  E  M  I  L  T  A  T  F  H  F  Y
421    TCAGAGCCGCCTCGGTGGCCTCGAGCGCTCGAGGTGCTATGCAAGCCGCGGGCCAAGAAC
137    S  E  P  P  R  W  P  R  A  L  E  V  L  C  K  P  R  A  K  N
481    GCTTCAGGCCGCCCGCTGCCCCTGGGCCCGCCCACACGCCAGCACCTGCTCTTCCGCAGC
157    A  S  G  R  P  L  P  L  G  P  P  T  R  Q  H  L  L  F  R  S
541    CTCTCGCAGAACACGGCCACACAGGGGCTACTCCGCGGGGCCATGGCCCTGGCGCCCCCA
177    L  S  Q  N  T  A  T  Q  G  L  L  R  G  A  M  A  L  A  P  P
601    CCGCGCGGCCTGTGGCAGGCCAAGGACATCTCCCCCATCGTCAAGGCGGCCCGCCGGGAT
197    P  R  G  L  W  Q  A  K  D  I  S  P  I  V  K  A  A  R  R  D
661    GGCGAGCTGCTCCTCTCCGCCCAGCTGGATTCTGAGGAGAGGGACCCGGGGGTGCCCCGG
217    G  E  L  L  L  S  A  Q  L  D  S  E  E  R  D  P  G  V  P  R
721    CCCAGCCCCTATGCGCCCTACATCCTAGTCTATGCCAACGATCTGGCCATCTCGGAGCCC
237    P  S  P  Y  A  P  Y  I  L  V  Y  A  N  D  L  A  I  S  E  P
781    AACAGCGTGGCAGTGACGCTGCAGAGATACGACCCCTTCCCTGCCGGAGACCCCGAGCCC
257    N  S  V  A  V  T  L  Q  R  Y  D  P  F  P  A  G  D  P  E  P
841    CGCGCAGCCCCCAACAACTCAGCGGACCCCCGCGTGCGCCGAGCCGCGCAGGCCACTGGG
```

MATCH WITH FIG. 1B

MATCH WITH FIG. IA

FIG. IB

```
277   R  A  A  P  N  N  S  A  D  P  R  V  R  R  A  A  Q  A  T  G
901   CCCCTCCAGGACAACGAGCTGCCGGGGCTGGATGAGAGGCCGCCGCGCGCCCACGCACAG
297   P  L  Q  D  N  E  L  P  G  L  D  E  R  P  P  R  A  H  A  Q
961   CACTTCCACAAGCACCAGCTGTGGCCCAGCCCCTTCCGGGCGCTGAAACCCCGGCCAGGG
317   H  F  H  K  H  Q  L  W  P  S  P  F  R  A  L  K  P  R  P  G
1021  CGCAAAGACCGCAGGAAGAAGGGCCAGGAGGTGTTCATGGCCGCCTCGCAGGTGCTGGAC
337   R  K  D  R  R  K  K  G  Q  E  V  F  M  A  A  S  Q  V  L  D
1081  TTTGACGAGAAGACGATGCAGAAAGCCCGGAGGAAGCAGTGGGATGAGCCGAGGGTGTGC
357   F  D  E  K  T  M  Q  K  A  R  R  K  Q  W  D  E  P  R  V  C
1141  TCCCGGAGGTACCTGAAGGTGGACTTCGCAGACATCGGCTGGAATGAATGGATAATCTCA
377   S  R  R  Y  L  K  V  D  F  A  D  I  G  W  N  E  W  I  I  S
1201  CCGAAATCTTTTGATGCCTACTACTGCGCGGGAGCATGTGAGTTCCCCATGCCTAAGATC
397   P  K  S  F  D  A  Y  Y  C  A  G  A  C  E  F  P  M  P  K  I
1261  GTTCGTCCATCCAACCATGCCACCATCCAGAGCATTGTCAGGGCTGTGGGCATCATCCCT
417   V  R  P  S  N  H  A  T  I  Q  S  I  V  R  A  V  G  I  I  P
1321  GGCATCCCAGAGCCCTGCTGTGTTCCCGATAAGATGAACTCCCTTGGGGTCCTCTTCCTG
437   G  I  P  E  P  C  C  V  P  D  K  M  N  S  L  G  V  L  F  L
1381  GATGAGAATCGGAATGTGGTTCTGAAGGTGTACCCCAACATGTCCGTGGACACCTGTGCC
457   D  E  N  R  N  V  V  L  K  V  Y  P  N  M  S  V  D  T  C  A
1441  TGCCGGTGAGACCACTCCAGGGTGGAAAGAAGCCACGCCCAGCAGAGCTGCCTTCTCGGA
477   C  R  *
1501  GCCTTCTGCAACCAGGACTTGTGGTGCAGCTGCAGACACAGAGCACAGCTCATGGGCAAC

1561  ATCACTGGGGCCCAGAGAGAGCTGTTCGCCAGTGCATCATTAGGGGGTCTTTCATTGCTT

1621  AGTGACTAGCCCCTTAAATG
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| Applicant's or agent's file reference number: 325800-405 | International application No.: PCT/US95/07915 |
|---|---|

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

**A.** The indications made below relate to the microorganism referred to in the description on pages 4, 33, (35), and 36 lines 2, 25, (47, 52), and 37, respectively.

**B. IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet ☐

Name of depository institution

American Type Culture Collection

Address of depositary institution (*including postal code and country*)

12301 Parklawn Drive
Rockville, Maryland 20852
U.S.A.

| Date of deposit: February 9, 1994 | Accession Number: 75672 |
|---|---|

**C. ADDITIONAL INDICATIONS** (*leave blank if not applicable*) This information is contained on an additional sheet ☐

**D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (*if the indications are not for all designated States*)

**E. SEPARATE FURNISHING OF INDICATIONS** (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

| For receiving Office use only | For International Bureau use only |
|---|---|
| ☐ This sheet was received with the international application | ☐ This sheet was received by the International Bureau on: 01 NOV 1996 |
| Authorized officer | Authorized officer |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07915

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.
US CL :530/399; 536/23.4; 435/6, 7.1, 7.2, 69.4, 240.2, 320.1; 514/1, 2, 44
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/399; 536/23.4; 435/6, 7.1, 7.2, 69.4, 240.2, 320.1; 514/1, 2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: BMP-10, bone morphogen

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US, A, 5,011,691 (OPPERMANN ET AL) 30 April 1991, see entire document. | 1-20 |
| A | JOURNAL OF CELL SCIENCE SUPPLEMENT, Volume 13, issued 1990, Wozney et al, "Growth factors influencing bone development", pages 149-155, see entire document. | 1-20 |
| A | MOLECULAR REPRODUCTION AND DEVELOPMENT, Volume 32, Number 2, issued June 1992, Wozney, "The Bone Morphogenetic Protein Family", pages 160-167, see entire document. | 1-20 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 08 SEPTEMBER 1995 | 25SEP1995 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | STEPHEN WALSH |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07915

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 184, Number 2, issued 30 April 1992, Burt, "Evolutionary Grouping of the Transforming Growth Factor-$\beta$ Superfamily", pages 590-595, see entire document. | 1-20 |
| A | GENES AND DEVELOPMENT, Volume 8, issued 1994, Kingsley, "The TGF-$\beta$ superfamily: new members, new receptors, and new genetic tests of function in different organisms", pages 133-146, see entire document. | 1-20 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07915

**B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07915

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 14/51; C12N 5/10, 15/18, 15/85; A61K 31/735, 38/18; C12Q 1/68; G01N 33/53, 33/567

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-11, 14 and 15, drawn to an isolated polynucleotide, a vector, an engineered host cell, a process for producing a polypeptide, a polypeptide, and methods of treatment.

Group II, claims 12 and 16, drawn to an agonist and a method of treatment.

Group III, claims 13 and 17, drawn to an antagonist and a method of treatment.

Group IV, claim 18, drawn to a process for diagnosing a disease by DNA sequence variation.

Group V, claim 19, drawn to a diagnostic process analyzing for a polypeptide.

Group VI, claim 20, drawn to a method for identifying compounds.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the compounds of Groups I, II and III have no structural or functional features in common and share no special technical features that are the same or corresponding. The compositions of Groups I, II or III are not used in or produced by the processes or methods of Groups IV, V or VI. The processes or methods of Groups I-VI have no process step that is the same or corresponding, and none of Groups I-VI use or produce any of the compositions of any other group. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.